Dev Genes Evol (2004) 214:261–266
DOI 10.1007/s00427-004-0403-7

SEQUENCE CORNER

**Marcel A. G. van der Heyden · Marleen van Eijk · Ronald Wilders · Jacques M. T. de Bakker · Tobias Opthof**

# Connexin43 orthologues in vertebrates: phylogeny from fish to man

Received: 5 February 2004 / Accepted: 7 March 2004 / Published online: 9 April 2004


**Abstract** The gap junction protein connexin43 (Cx43) is widely expressed in all vertebrate species; however, in ventricular myocardium, Cx43 expression is restricted to mammalian species only, where it provides the molecular correlate for both electrical conduction and synchronization of the repolarization process. The evolutionarily "late" appearance of Cx43 in the heart suggests physiological adaptation to euthermia with its concomitant demands related to increased cardiovascular output. We tested to what extent mammalian Cx43 differs from that of non-mammalian vertebrates and whether Cx43 from hibernating species contains specific sequence characteristics which could be attributed to their non-isothermal life cycle. We cloned the complete coding region of Cx43 from the African green monkey, European hedgehog (hibernator), Russian dwarf hamster, rabbit, European ground squirrel (hibernator) and pig. After sequencing, these were compared to 12 full-length Cx43 sequences present in GenBank (3 fish, 2 frogs, chicken and 6 mammals amongst which there was one other hibernator). Overall identity ranged from 68.7% to 97.7% at the nucleotide level and from 71.6% to 99.7% at the amino acid level. The phylogeny of Cx43 mirrors the general phylogenetic histories of the investigated species to a large extent. From 382 amino acids there were only 6 specific for mammals. There were no substitutions specific for hibernators. In conclusion, mammalian Cx43 is characterized by 6 specific amino acids, and no obvious differences between non-hibernating and hibernating mammals were observed.

**Keywords** Gap junction · Connexin · Sequence · Evolution

Edited by D. Tautz

M. A. G. van der Heyden (✉) · M. van Eijk · J. M. T. de Bakker · T. Opthof
Department of Medical Physiology,
University Medical Center Utrecht,
PO Box 85060, 3508 AB Utrecht, The Netherlands
e-mail: m.a.g.vanderheyden@med.uu.nl
Tel.: +31-30-2538418
Fax: +31-30-2539036

R. Wilders
Department of Physiology, Academic Medical Center,
University of Amsterdam,
Amsterdam, The Netherlands

## Introduction

Gap junctions provide the path for direct exchange of small molecules and ions (and current) between adjacent cells. They are built of innexins as pore-forming units in invertebrates (Phelan and Starich 2001) and of connexins in vertebrates (Goodenough and Paul 2003) and whereas their topology is similar, they present no sequence homology. In vertebrates, six connexins associate to form one hemichannel (connexon), and subsequent docking of two connexons in adjacent cell membranes results in the formation of a gap junction. To date, 21 different connexin isoforms have been described in the human genome (Willecke et al. 2002; Söhl and Willecke 2003). Of these, connexin43 (Cx43) displays a very broad and mainly conserved expression pattern in vertebrate species, i.e. in the lens epithelium of frogs (van der Heyden et al. 2001), chicken (Musil et al. 1990) and rat (Beyer et al. 1989). In the working myocardium, however, Cx43 expression is restricted to mammalian species (Becker et al. 1998). In the mammalian heart, Cx43 is vital for both conduction of the ventricular action potential and synchronization of the ventricular repolarization process.

The hibernator's heart carries several intriguing enigmas with potential relevance to future understanding of arrhythmogenesis. The heart of a hibernating animal remains electrically and mechanically active at temperatures which are incompatible with life in other mammals either due to asystole or to induction of ventricular fibrillation (Johanssen 1967). This suggests specialization during evolution with respect to membrane currents relevant for ventricular repolarization including the synchronization of this process mediated by gap junctions.

**Table 1** Species abbreviations and GenBank accession numbers of connexin43 molecules described in this study

| Abbr | Species | Common name | GenBank accession number |
|---|---|---|---|
| Bt | *Bos taurus* | Bovine | J05535 |
| Ca | *Cercopithecus aethiops* | African green monkey/COS | AY382588, this study |
| Cc | *Cyprinus carpio* | Common carp | AY008286 |
| Cg | *Cricetulus griseus* | Chinese dwarf hamster | AY206456 |
| Da | *Danio aequipinnatus* | Giant danio | AF067407 |
| Dr | *Danio rerio* | Zebrafish | AF035481 |
| Ee[a] | *Erinaceus europaeus* | European hedgehog | AY382589, this study |
| Gg | *Gallus gallus* | Chicken | M29003 |
| Hs | *Homo sapiens* | Human | M65188 |
| Ma[a] | *Mesocricetus auratus* | Syrian hamster | AY206455 |
| Mm | *Mus musculus* | Mouse | X61576 |
| Oc | *Oryctolagus cuniculus* | Rabbit | AY382590, this study |
| Ps | *Phodopus sungorus* | Russian dwarf hamster | AY382591, this study |
| Rn | *Rattus norvegicus* | Norwegian rat | X06656 |
| Sc[a] | *Spermophilus citellus* | European ground squirrel | AY382592, this study |
| Ss | *Sus scrofa* | Pig | AY382593, this study |
| Xl | *Xenopus laevis* | African clawed frog | X17243 |
| Xt | *Xenopus tropicalis* | West-African clawed frog | AY043270 |

[a] Hibernator

The evolutionarily "late" appearance of Cx43 in the myocardium may point to physiological adaptation to euthermia with its concomitant demands related to a more intense energy metabolism and increased cardiovascular output. Thus, we questioned (1) which amino acids distinguish mammalian Cx43 from other vertebrate Cx43 proteins and whether (2) hibernating species display specific sequence characteristics within the Cx43 protein as a possible evolutionary adaptation to their non-isothermal life cycle. To this end we cloned the full-length coding region of Cx43 from 6 mammalian species. These were compared to 12 full-length Cx43 sequences from GenBank, including fish, amphibians, bird and other mammals. Our analysis included three hibernating species (European hedgehog, European ground squirrel and Syrian hamster).

## Materials and methods

Genomic DNA was isolated by incubating tissue samples (Ee, Oc, Ps, Sc, Ss, all muscle; Ca, cultured cells) in lysis buffer [100 mM Tris/HCl (pH 8.0), 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 500 $\mu$g/ml proteinase K] for 16 h at 56°C. The supernatant was phenol/chloroform extracted twice and genomic DNA was precipitated using ethanol. Subsequently, Cx43 was amplified using a mix of two sense and three antisense primers (sense ATGGGTGACTGGAGCGCCTT and ATGGGTGACTGGAGTGCCTT, antisense ATCTCCAGGTCATCAGGCCG, ATCTCTAGGTCATCAGGCCG and ATCTCCAAATCATCAGGTCG) using an annealing temperature of 56°C and 30–35 amplification cycles. The PCR products were analyzed and isolated from 1% agarose/TAE ethidium bromide stained gels using JetSorb (Genomed) and ligated into pGEM-T-easy (Promega). Cx43 was subsequently sequenced using universal Sp6 and T7 primers and Cx43-specific internal primers. Cloning of Cx43 from each species was performed twice, independently. Sequencing of both clones raised identical results.

Alignment and phylogenetic tree construction of new and full-length Cx43 sequences from GenBank was performed with Lasergene software (DNASTAR, Madison, Wis.), operating with ClustalW and Joint Neighbour algorithms (Saitou and Nei 1987; Higgins and Sharp 1990).

## Results and discussion

To isolate Cx43 sequences from various species, we aligned nucleotide sequences of mouse, rat and human Cx43 and subsequently designed a primer set consisting of two sense and three antisense oligonucleotides encompassing the entire protein coding region. To test the ability to amplify novel Cx43 sequences, PCR was performed on genomic DNA from human (Hs), mouse (Mm), Russian dwarf hamster (Ps) and pig (Ss; see also Table 1). Figure 1a shows amplification of a PCR product of ~1,150 bp from all templates. Digestion with *Eco*RI (Fig. 1b) and *Sac*I (Fig. 1c) gave the predicted results for human and mouse PCR products and identical-sized fragments for Russian dwarf hamster and pig. These results demonstrate the ability of this primer set to amplify Cx43 from several different mammalian species.

Subsequently, Cx43 was amplified and cloned from African green monkey (Ca), European hedgehog (Ee), European ground squirrel (Sc), pig, Russian dwarf hamster and rabbit (Oc; see Table 1 for GenBank accession numbers). Following sequencing, alignments were performed including 12 previously described Cx43 sequences. Percentages of identity at the nucleotide level are between 68.7% (giant danio vs West African clawed frog: Da vs Xt) and 97.7% (Hs vs Ca). Identity percentages are higher at amino acid level and are between 71.6% (Da vs Xl/Xt) and 99.7% (Syrian hamster vs Russian dwarf hamster: Ma vs Ps). Figure 2 shows the result of nucleotide phylogenetic analysis. This analysis yields a clear distinction between the classes of mammals, fish, amphibia and birds and also clearly defined relationships within the classes (i.e. order of primates or suborder of hamsters).

Figure 3 reveals two main regions of dissimilarity in amino acid alignment between the Cx43 from various species. The first region is located in the intracellular loop, which is located between transmembrane domains 2 and 3; the second one follows the fourth transmembrane

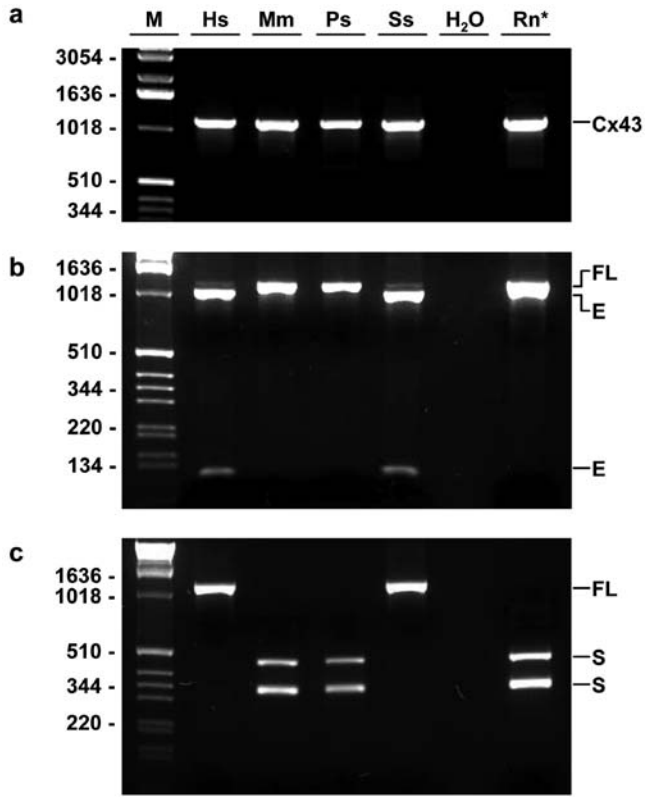



**Fig. 1a–c** Amplification of Cx43 from genomic DNA by PCR. **a** Full length product; **b**, **c** PCR product digested with *Eco*RI or *Sac*I, respectively. Position of the marker (*M*) is indicated at the left (base pairs). Species abbreviations are as in Table 1. *Lane Rn** Positive control using rat Cx43 cDNA in pBluescript vector, $H_2O$ negative control; *FL* full length product, *E Eco*RI digestion product, *S Sac*I digestion product

region and is thus located in the intracellular C-terminus. Currently, many residues within Cx43 have been linked to a certain function. The six cysteine residues within the two extracellular loops, which are found in all connexin protein family members, have been implicated in intercellular docking of connexons (Dahl et al. 1992). These residues are conserved between all species. Tyrosine 265 has been mapped as a Src phosphorylation site (Swenson et al. 1990) and is conserved between all species. Of the three potential PKC phosphorylation sites in the C-terminus (Kanemitsu and Lau 1993; Sáez et al. 1997; Lampe et al. 2000), S365 and S369 are conserved between all species, and hence are probably the most important. In rat, the charged amino acids R243 and D245 are implicated in membrane potential dependency of the gap junction channel (Revilla et al. 2000). Of these sites, R243 is changed into the positively charged amino acid K in many other species, indicating the importance of a positive charge at this position. The negatively charged D at 245 is changed to N in fishes only, and is conserved in all other species. Upon intracellular acidification, Cx43-based gap junctions will close (Morley et al. 1997). It is proposed that this so-called pH dependent gating results from intramolecular interactions of the C-terminus with other parts of the protein. The conserved H95 appears to play a role (Ek et al. 1994), and more recently other regions have been mapped. In the intracellular loop, two short domains are involved (rCx43 N122-L127 and I139-G143) of which the second one is well conserved (Duffy et al. 2002).

Many mutations in connexins have been linked to specific diseases (Söhl and Willecke 2003). Most of the 24 Cx43 mutations found thus far in humans, which very often result in a pleiotropic phenotype (Paznekas et al. 2002), are located at very conserved sites suggesting a general role in gap junction function, rather than a tissue-specific function. Of the four single nucleotide polymorphisms presented in the NCBI database which result in amino acid substitutions (R148Q, A168T, R202C, T204 M) the last two are conserved between all species. A168 is conserved in mammals and chicken. Interestingly, R148 is a conserved charge at this position, being K in fish and R in all other species, and thus could have a physiological role. Mutational analysis of these sites and/

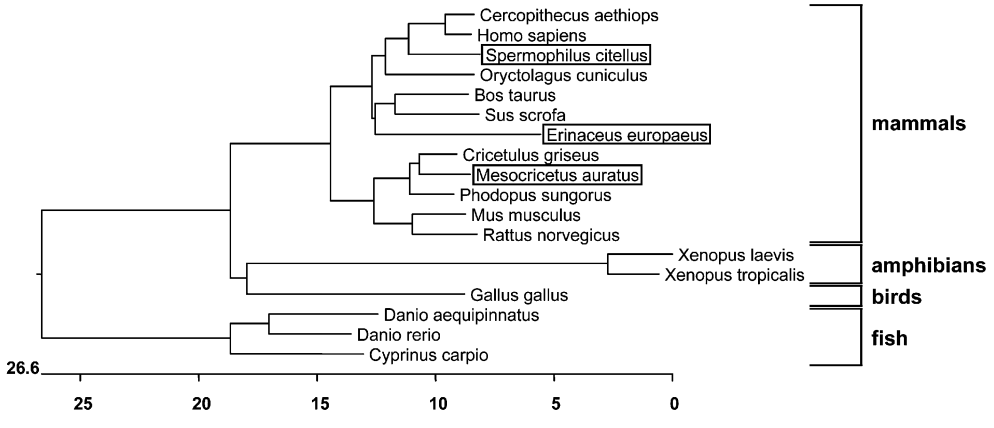
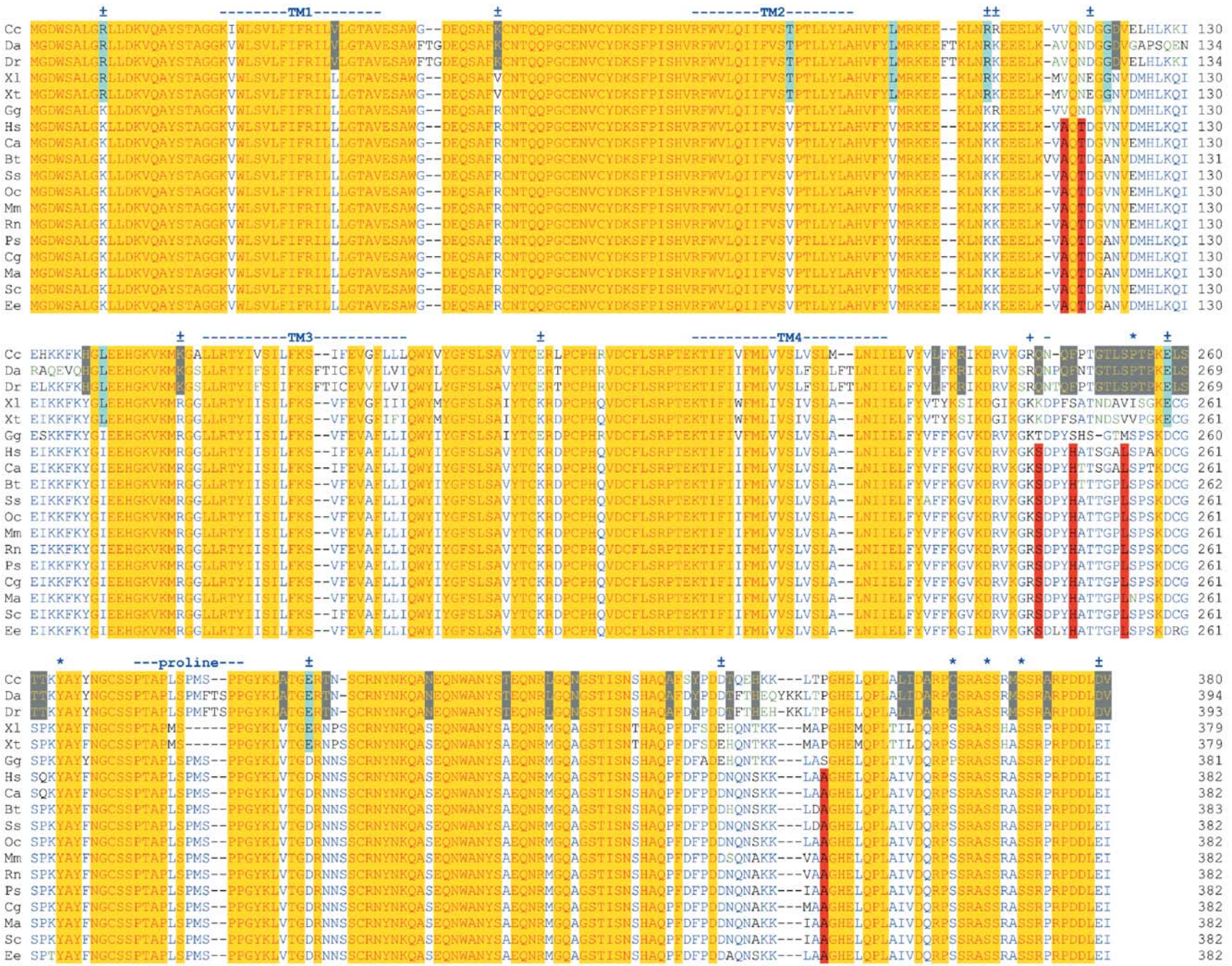



**Fig. 2** Cladogram of Cx43 sequences. The nucleotide sequences of the protein coding region of 18 Cx43s from fish, birds, amphibians and mammals were analyzed using the Clustal method of the Megalign program of the Lasergene software package DNASTAR. *The scale beneath the tree* measures the evolutionary distance between the sequences, and *units* indicate the number of substitution events. Hibernating species are *boxed*


TM1
TM2
TM3
TM4
proline

or genetic screening on diverse patient groups may result in identifying a role for these amino acids. Many amino acid changes demarcate the border between fish and other classes of the vertebrates (i.e. V36 vs L36), or between fish/amphibians and the other classes (i.e. R9 vs K9). Only six mammalian specific amino acid substitutions were observed. These substitutions demarcate the evolutionary border between the mammals and fish/amphibians/birds (with and without connexin43 in the ventricular working myocardium). These sites are shaded red (Fig. 3 Hs: A116, T118, S244, H248, L254, A349); three of these (T118, S244 and H248) are of particular interest because they may be phosphorylated (T/S) or charged (H). Interestingly, S244 is positioned within the region of the proposed membrane potential voltage sensor (Revilla et al. 2000). No relationship was found between hibernation and Cx43 coding sequences. With such a limited amount of mammalian-specific amino acids, it is not a surprise that there are no hibernator-specific amino acids (European hedgehog, European ground squirrel, Syrian hamster). On the other hand, pleiotropy might have a role as well (Griswold and Whitlock 2003). Since Cx43 is broadly expressed in many different tissues and organs, Cx43 amino acid substitutions that are beneficial for cardiac performance during hibernation might be deleterious in other organs, and therefore selected against. We suggest that in the working myocardium Cx43 serves as a gross channel of which the intrinsic properties do not allow the required fine-tuning of conduction velocity and repolarization in specific species such as hibernators. We suggest that other forms of regulation, i.e. transcriptional and post-translational regulation of Cx43 expression, or direct interactions with other proteins (reviewed in Giepmans 2004) are more important in encountering the physiological needs of a given species under specific conditions.

**Acknowledgements** We thank Dr. R.H. Henning and Dr. L.E. Deelman (Groningen University, the Netherlands), Dr. J. Seddon (Uppsala University, Sweden) and Dr. G. Hewitt (University of East Anglia, United Kingdom), M.C.J. Mutsaers (Super de Boer, Princehage), and P. Vrolijk (Hamstery Vrolijk, Nieuwegein) for providing tissue samples from ground squirrel, hedgehog, pig and Russian dwarf hamster, respectively. This study was supported by STW grant MKG.5942 (MvdH).

**Fig. 3** Amino acid alignment of 18 full length Cx43s. Species abbreviations are as in Table 1. *TM* Transmembrane domain (there are four domains separated by two extracellular loops and one intracellular loop), *Proline* proline repeat, * experimentally confirmed phosphorylation site, + *and* − charged residues experimentally confirmed to be involved in gating, ± conserved charge, *yellow shaded* conserved between all species, *red shaded* conserved between mammals, *grey shaded* conserved between fish, *blue shaded* conserved between fish and frogs

## References

Becker DL, Cook JE, Davies CS, Evans WH, Gourdie RG (1998) Expression of major gap junction connexin types in the working myocardium of eight chordates. Cell Biol Intl 22:527–543

Beyer EC, Kistler J, Paul DL, Goodenough DA (1989) Antisera directed against connexin43 peptides react with 43-kDa protein localized to gap junctions in myocardium and other tissues. J Cell Biol 108:595–605

Dahl G, Werner R, Levine E, Rabadan-Diehl C (1992) Mutational analysis of gap junction formation. Biophys J 62:172–180

Duffy HS, Sorgen PL, Girvin ME, O'Donnell P, Coombs W, Taffet SM, Delmar, Spray DC (2002) pH-dependent intramolecular binding and structure involving Cx43 cytoplasmic domains. J Biol Chem 39:36706–36714

Ek JF, Delmar M, Perzova R, Taffet SM (1994) Role of histidine 95 in pH gating of the cardiac gap junction protein connexin 43. Circ Res 74:1058–1064

Giepmans BNG (2004) Gap junctions and connexin-interacting proteins. Cardiovasc Res. DOI 10.1016/j.cardiores.2003.12.009

Goodenough DA, Paul DL (2003) Beyond the gap: functions of unpaired connexon channels. Nat Rev Mol Cell Biol 4:285–294

Griswold CK, Whitlock MC (2003) The genetics of adaptation: the roles of pleiotropy, stabilizing selection and drift in shaping the distribution of bidirectional fixed mutational effects. Genetics 165:2181–2192

Heyden MAG van der, Roeleveld L, Peterson J, Destrée OHJ (2001) Connexin43 expression during *Xenopus* development. Mech Dev 108:217–220

Higgins DG, Sharp PM (1990) Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl Biosci 5:151–153

Johansson BW (1967) Heart and circulation in hibernators. In: Fisher KC, Dawe AR, Lyman CP, Schönbaum E, South FE (eds) Mammalian hibernation III. Oliver and Boyd, Edinburgh, pp 199–218

Kanemitsu MY, Lau AF (1993) Epidermal growth factor stimulates the disruption of gap-junctional communication and connexin43 phosphorylation independent of 12-O-tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. Mol Biol Cell 4:837–848

Lampe PD, TenBroek EM, Burt JM, Kurata WE, Johnson RG, Lau AF (2000) Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. J Cell Biol 149:1503–1512

Morley GE, Ek-Vitorín JF, Taffet SM, Delmar M (1997) Structure of connexin43 and its regulation by $pH_i$. J Cardiovasc Electrophysiol 8:939–951

Musil LS, Beyer EC, Goodenough DA (1990) Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization, and post-translational phosphorylation. J Membr Biol 116:163–175

Paznekas WA, Boyadjiev SA, Shapiro RE, Daniels O, Wollnik B, Keegan CE, Innis JW, Dinulos MB, Christian C, Hannibal MC, Jabs EW (2003) Connexin43 (GJA1) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. Am J Hum Genet 72:408–418

Phelan P, Starich TA (2001) Innexins get into the gap. BioEssays 23:388–396

Revilla A, Bennett MV, Barrio LC (2000) Molecular determinants of membrane potential dependence in vertebrate gap junction channels. Proc Natl Acad Sci USA 97:14760–14765

Sáez JC, Nairn AC, Czernik AJ, Fishman GI, Spray DC, Hertzberg EL (1997) Phosphorylation of connexin43 and the regulation of neonatal rat cardiac myocyte gap junctions. J Mol Cell Cardiol 29:2131–2145

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Söhl G, Willecke K (2003) An update on connexin genes and their nomenclature in mouse and man. Cell Commun Adhes 10:173–180

Swenson KI, Piwnica-Worms H, McNamee H, Paul DL (1990) Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-src-induced inhibition of communication. Cell Regul 1:989–1002

Willecke K, Eiberger J, Degen J, Eckardt D, Romualdi A, Guldenagel M, Deutsch U, Söhl G (2002) Structural and functional diversity of connexin genes in the mouse and human genome. Biol Chem 383:725–737